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BURKHOLDERIA CEPACIASTRAIN IISRCLRB5 MEDIATED INDUCTION OF DEFENCE RELATED ENZYMES AND PHENOLIC COMPOUNDS TO ENHANCE THE RESISTANCE IN TURMERIC (CURCUMA LONGA L.) TO PYTHIUMAPHANIDERMATUM

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ABSTRACT

Burkholderia cepacia IISRCLRB5 was found to be antagonistic to the rhizome rot pathogen Pythium aphanidermatum in turmeric both in vitro and in vivo. In this study the strain was tested for its ability to induce defense related enzymes viz, peroxidase (PO), poly phenol oxidase (PPO), phenylalanine ammonia lyase (PAL), lipoxygenase (LOX), β(1,3) glucanase and total phenols against P. aphanidermatumin turmeric plants. The application of rhizobacteria two days before challenge inoculation with P. aphanidermatum recorded the highest induction of all the tested defense related enzymes (PO, PPO, PAL, LOX, β (1,3) glucanase and accumulation of phenolics compared with individual application of pathogen, rhizobacterium and untreated plants. These results suggest that the increased induction of defense enzymes involved in phenyl propanoid pathway and accumulation of phenolics and PR-proteins might have contributed to restriction of invasion of Pythium aphanidermatum in turmeric plants.

KEYWORDS: Bhurkolderia cepacia, Biocontrol Agent, Curcuma longa L, IISRCLRB5, Defence Enzymes, P. aphanidermatum, Peroxidase, Poly Phenol Oxidase, Phenylalanine Ammonia Lyase, Lipoxygenase, B (1,3) glucanase & Total Phenols

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Original Article

1. INTRODUCTION

One of the important constraints which limit the production of turmeric in India is rhizome rot diseases. Eco friendly management strategies such as the use of bio agents are of much importance due to the ecological hazards inflicted by the plant protection chemicals on its excessive use. 'Plant growth promoting rhizobacteria' (PGPR), are known to induce systemic resistance in the host plant, which may reduce the disease incidence and increase the yield (Haas and Défago, 2005). A state of enhanced defensive capacity developed by a plant when appropriately stimulated by microorganism or environmental stress is referred as induced systemic resistance (Zhu-Salzman *et al*, 2005). The selected plant growth-promoting bacteria in the rhizosphere prime the whole plant for enhanced defense against a broad spectrum of pathogens in Induced Systemic Resistance (Pieterse *et al*, 2014). ISR usually lasts for relatively long time in plants and the level may change after initial elicitation (Kuc, 2001). Defence enzymes associated with ISR includes, phenylalanine ammonia lyase (PAL), peroxidase (PO), poly phenol oxidase (PPO), catalase (CAT), lipoxygenase, β- 1,3 glucanase, chitinase, superoxide dismutase (SOD), ascorbate peroxidase (APX) and proteinase inhibitors (van Loon, 1997), may bring about the liberation of molecules which elicit first line of resistance induction, phenolics and phytoalexins (Keen and Yoshikawa, 1983). ISR exhibited by biocontrol has been established as a potential tool in crop protection against diseases (Manikandan and Raguchander, 2014).

Prior application of biological inducer is a novel technique for inducing plant's own defence mechanism which confers plant protection (Kuc, 1995). The bacteria *Burkholderia cepacia* strain IISRCLRB5 isolated from healthy turmeric rhizosphere have been reported to protect turmeric plants against pathogenic rhizome rot pathogen *Pythium aphanidermatum* through a range of mechanisms, including antagonism via siderophore production, IAA production, ammonia production, phosphate solubilization, potassium utilization, α- amylase production and protease production (Bijitha and Suseela, 2019). In this contest, it is pertinent to explore the potentiality of *Burkholderia cepacia* IISRCLRB5 in inducing the systemic resistance against the rhizome rot pathogen *Pythium aphanidermatum*. The study encompasses to understand the mechanism of *B. cepacia* IISRCLRB5 induced systemic resistance through defence related enzymes activity and phenolic compounds in turmeric against rhizome rot pathogen.

2. MATERIALS AND METHODS

Turmeric Material: Disease free rhizomes of turmeric variety 'IISR Prathibha' were procured, grown and maintained in sterile porting mixture (soil: sand: cow dung, 2:2:1) in pro trays under greenhouse conditions. 45 days old plants were used for the study.

Biocontrol Agent (Rhizobacterium): 1 ml log phase culture of (3×10^{10} CFU mL^{-1}) of *B. cepacia* strain IISRCLRB5 was added to the nutrient broth and incubated at room temperature (25 ± 2 °C) for 48 hours and used for the study.

Pathogen: *P. aphanidermatum* grown for 72 h on PDA plates was macerated and 10 mL of the concentrate (1g mycelium) was then suspended in 100 mL of distilled water served as the pathogen inoculum**.**

Treatments: Four treatments were included in the study with three replications in a completely randomized manner on variety IISR- Prathibha. The treatments were T1- absolute control, T2- pathogen control (challenged with *Pythium aphanidermatum* inoculum), T3- rhizobacterium control (plants treated with the bacterial suspension alone) and T4- *rhizobacterium* + pathogen (pathogen inoculated 2 days after rhizobacterium treatment).

Sampling: Plants were carefully uprooted without causing any damage to rhizome tissues at different time intervals from 0 (1 hour after pathogen challenging) to 8 days post inoculation (DPI) at 24 h intervals. The fresh plants were washed in running tap water, the leaf, rhizome and root tissues were drawn, weighed and ground in respective buffer solutions using a pre-chilled mortar and pestle and subjected to biochemical analyses for peroxidase, polyphenol oxidase, phenyl alanine ammonia lyase, lipoxygenase, β (1,3) glucanase and phenols.

Peroxidase (PO) Assay

Peroxidase activity was performed according to Jennings *et al*, (1969). Samples were extracted in 1 mL of 50 mM Tris-HCl buffer (pH 7.5) by grinding at 4°C. The homogenate was centrifuged at 13,000 g for 20 min at 4°C and the supernatant was used for the enzyme assay. The reaction mixture consisted of 0.5 mL of the enzyme extract, 0.5 mL of the substrate 1% guaiacol, 0.5 mL of 1% H_2O_2 and 1.5 mL of 50 mM Tris- HCl buffer (pH 7.5). In a spectrophotometer, the peroxidase activity was determined by measuring the change in absorbance at 20 s interval for a period of 3 min at 470 nm. The enzyme activity was calculated using the extinction coefficient for tetraguaiacol (26600 M^{-1} cm^{-1}) and was expressed in micromoles of tetraguaiacol formed per min per gram fresh weight. Tris- HCl buffer served as blank.

Poly Phenol Oxidase (PPO) Assay

The PPO assay was performed spectrophoto metrically using Mayer *et al*, (1966) with slight modifications. Respective

plant tissue samples were homogenized in 1 mL of 0.1 M sodium phosphate buffer (pH 6.5) with 0.1 % Triton-X and centrifuged at 13,000 g for 20 min at 4°C and the supernatant was used for the assay. 2.5 mL sodium phosphate buffer and 0.5 mL enzyme extract constituted the reaction mixture, into which 200 µL of 0.01 M catechol was added. At 495 nm, the change in absorbance with the formation of catechol quinone was noted at 20 s interval for 3 min. The extinction coefficient for oxidized catechol (3.6×10^4 $M^{-1}cm^{-1}$) as micromoles of substrate converted per min per gram fresh weight provides the enzyme activity.

Phenylalanine Ammonia Lyase (PAL) Assay

The PAL assay was determined using Peltonen and Karjalainen, (1995) method. The respective plant tissue samples were homogenized in 1 mL of 50 mM Tris- HCl buffer (pH 8.5) at 4^0C, the homogenate was centrifuged at 13,000 g for 10 min at 4^0C. The supernatant was used for enzyme activity assay. 2.9 L of 0.2 % L- phenylalanine in 50 mM tris- HCl (pH 8.5) and 0.1 mL of enzyme extract constituted the reaction mixture. The mixture was incubated for 1 h at 37^0C and the optical density was recorded at 290 nm. The amount of trans- cinnamic acid produced was calculated as the micromoles of trans-cinnamate produced per minute per gram fresh weight using a standard curve developed for trans-cinnamic acid.

Lipoxygenase (LOX) Assay

The enzyme assay was performed according to the method of Plagemann *et al*, (2013) with slight modifications. Samples of respective plant tissues were ground in 1 mL of 0.1 M sodium phosphate buffer (pH 6.5) at 4^0C. The homogenate was centrifuged at 13,000 rpm for 20 min at 4^0C and the supernatant was used as the enzyme source. The reaction mixture constitutes 3 mL of 0.1 M sodium phosphate buffer, 0.05 mL of enzyme extract and 0.75 mL of freshly prepared solution of linoleic acid substrate (buffered). The enzyme activity was obtained by monitoring the change in absorbance at 234 nm for 3 min and the enzyme activity was expressed as micromoles of substrate converted per minute per gram fresh weight.

Beta-1, 3 Glucanase Assay

Beta-1, 3-glucanase activity was assayed spectrophotometrically using the Nelson Somogyi method (Somogyi, 1952) with slight modifications. Respective plant tissues were extracted with 1 mL of 0.1M sodium acetate buffer (pH 5.0) at 4^0C. The homogenate was centrifuged at 10,000 rpm for15 min at 4^0C, the supernatant served as the crude enzyme extract for the assay. 0.075 mL enzyme extract and 0.075 ml substrate, 4% laminarin constituted the reaction mixture. The mixture was incubated for 10 min at 40^0C and then stopped by the addition of alkaline copper reagent and boiled for 10 min on a water bath. Beta-1, 3 glucanase activity was determined by measuring absorbance at 620 nm in a spectrophotometer. The enzyme activity was compared against glucose standards and expressed in units of micromoles of substrate converted per minute per gram fresh weight.

Estimation of Phenols

Total phenols were extracted according to Folin- Ciocalteau method described by Gutfinger, 1981 with slight modifications. Respective plant tissues were ground in 1.5 mL of 80 % methanol at room temperature. The extracts were centrifuged and the supernatant was used for the assay of total phenols. About 0.5 mL of Folin-Ciocalteau reagent was added to 0.2 mL of the extract, after 3 min, 1 mL of saturated sodium carbonate solution was added and diluted to 10 mL volume with distilled water. After 1 h, the absorbance was read at 725 nm in a spectrophotometer and quantified against gallic acid standard.

Statistical Analysis

The significance of treatment effects was determined by one-way analysis of variance (ANOVA) with the statistical package SAS software (Version 9.3) and subjected to mean separation by the Least Significant Difference (LSD) test, $p < 0.05$.

RESULTS

Results of spectrophotometric assay revealed that application of rhizobacterium significantly increased the activities of total phenols and defence enzymes *viz.*, PO, PPO, PAL, LOX and β 1, 3 glucanase in turmeric leaf, rhizome and root tissues against the rhizome rot pathogen.

Assay of Peroxidase (PO)

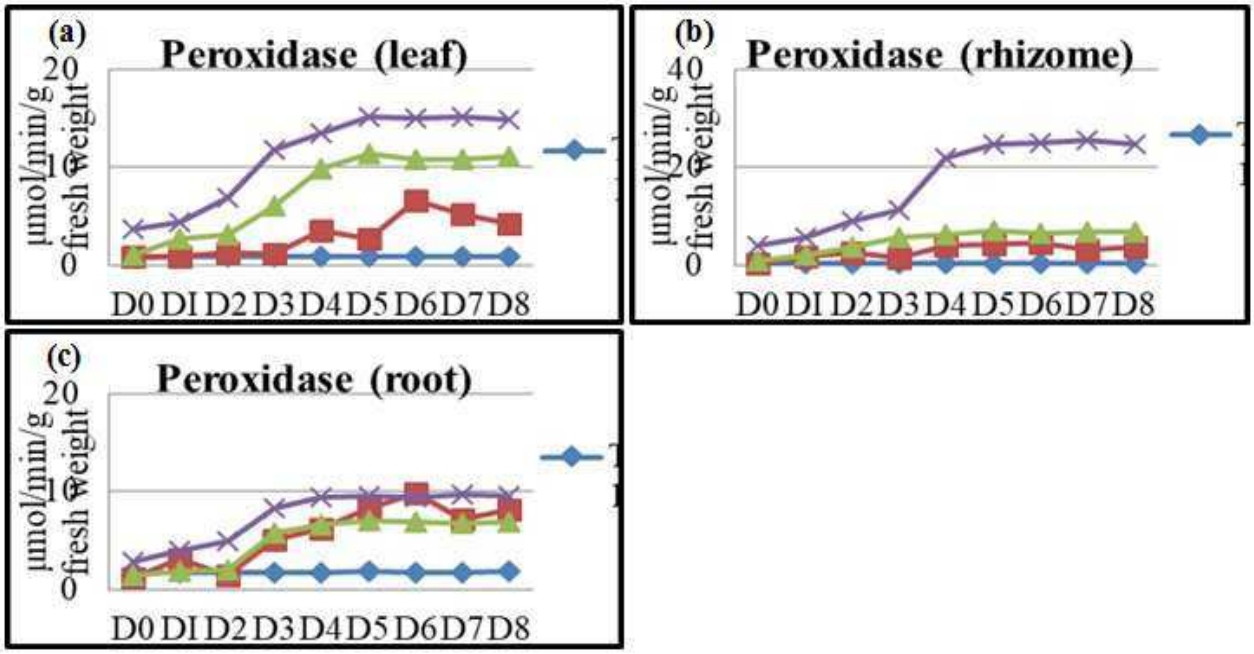

Figure 1: Change in PO Activity in; (A)- Leaf, (B)- Rhizome and (C)- Root Tissues of Turmeric Treatments; DPI- Days Post Inoculation; T1-Absolute Control, T2- Pathogen Control T3- Rhizobacterium Alone and T4- *Rhizobacterium* + Pathogen; D0 to D8- Day 0 to Day 8 (LSD at 5% Level of Significance).

Significant difference in peroxidase activity could not be observed under uninoculated condition. Upon challenging with *P. aphanidermatum*, on 1st day itself significant, enhanced enzyme activity was shown by both T3 and T4 in all the tissues, while the highest activity was recorded with T4. In T4, the leaf tissues showed the highest activity from day 5 onwards and the enzyme activity remained significantly high throughout the study period. On day 5 the activity was 18.03 folds higher than that of absolute control plants (T1) and 5.69 folds higher than that of T2. It was observed that the enzyme activity in T3 is 13.45 folds higher than that of absolute control plants (T1). In rhizome tissues also, the highest peroxidase activity was observed with T4 lines. In root tissues, the highest peroxidase activity was noticed on day 6 of T2 (11.46 folds higher) and it was higher than that of the highest value in T4 lines but thereafter gradually decreased. T4 plants also recorded high peroxidase activity from day 3 onwards. The highest activity of 10.87 folds higher value was observed on day 7 and remains at par from day 4 onwards in T4.

Assay of Poly Phenol Oxidase (PPO)

Like peroxidase, there is no significant difference in poly phenol oxidase activity was observed under uninoculated condition (T1). Upon pathogen inoculation, enzyme activity increased in all the three tissues of all the treatment lines, being the highest activity on T4. In leaf tissues, maximum activity was observed from day 5 up to day 8 in T4 of which the highest activity marked on day 6 (5.43 folds). In T3 also a marked increase was noted on day 5 onwards (around 4.47 folds). In T2 also, an increase in activity was noted with highest value on day 5 (3.64 folds) but it has got decreased afterwards. In rhizome tissues also a marked increase in activity was noted in T4 with highest activity on day 8 with 12.23 folds increase as compared to the T1. Rhizome tissues of T3 plants also showed significant increase in enzyme activity followed by T2 plants. In root tissues also highest PPO activity was observed with T4, highest being on day 8 with 14.53 folds increase followed by T3 plants with highest value on day 5 with 8.81 folds increase. T2 plants also noticed a slight increase in activity from day 0 to day 6 while the activity decreased significantly from day 7 onwards.

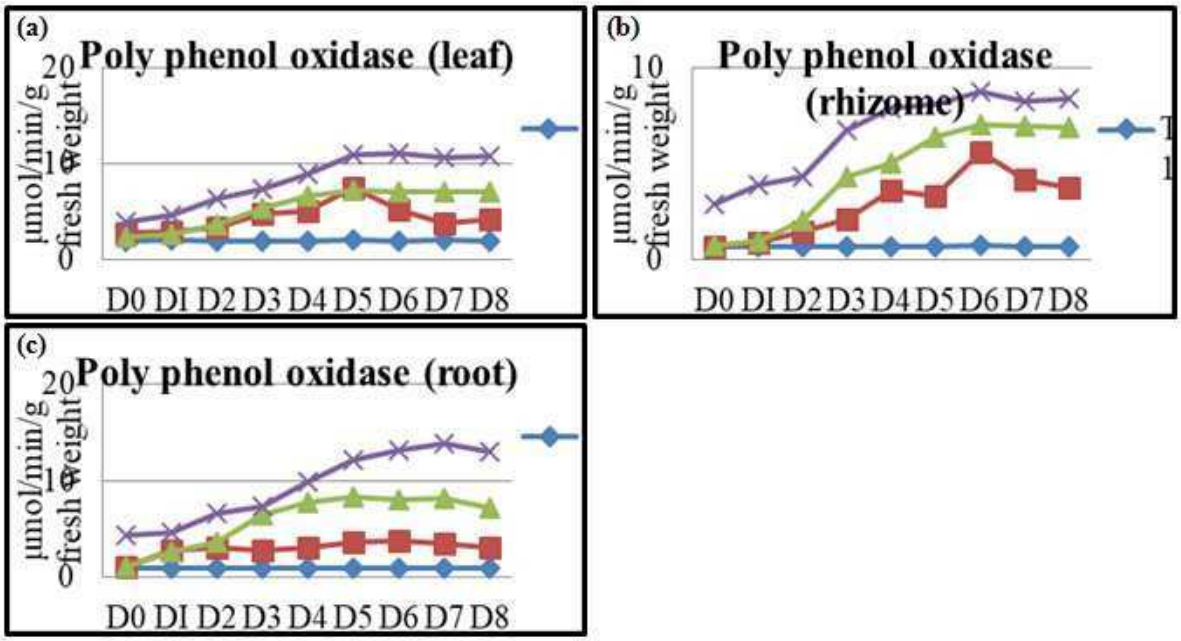

Figure 2: Change in PPO Activity in;(A)- Leaf, (B)- Rhizome and (C)- Root Tissues Of Turmeric Treatments; DPI- Days Post Inoculation; D0 To D8- Day 0 To Day 8 (LSD at 5% Level of Significance).

Assay of Phenylalanine Ammonia Lyase (PAL).

As in earlier cases, there is no significant difference in poly phenol oxidase activity was observed under uninoculated condition (T1). In leaf tissues, highest activity of PAL was noticed on day 6 of T3 (8.59 folds) followed by T2 (8.33 folds). T4 also recorded a significant increase in activity with highest values on day 7(7.26 folds), 4 (6.65 folds) and 8 (6.64 folds). In rhizome tissues, significantly high activity was noted from day 0 onwards with highest activity on day 6 (7.26 folds) and 8 (6.64 folds) in T4. As in rhizome tissues, root tissues also showed enhanced values of PAL activity on T4 from day 0 onwards, high values being observed with highest activity on day 5 (9.18 folds) and remained slightly similar throughout the experiment period. In T3, significant increase was noted from day 1 with highest activity on day 8 (8.2 folds). Even though T2 recorded higher activity in all the tissues, a significant decrease was noted in all the tissues towards the end of experiment.

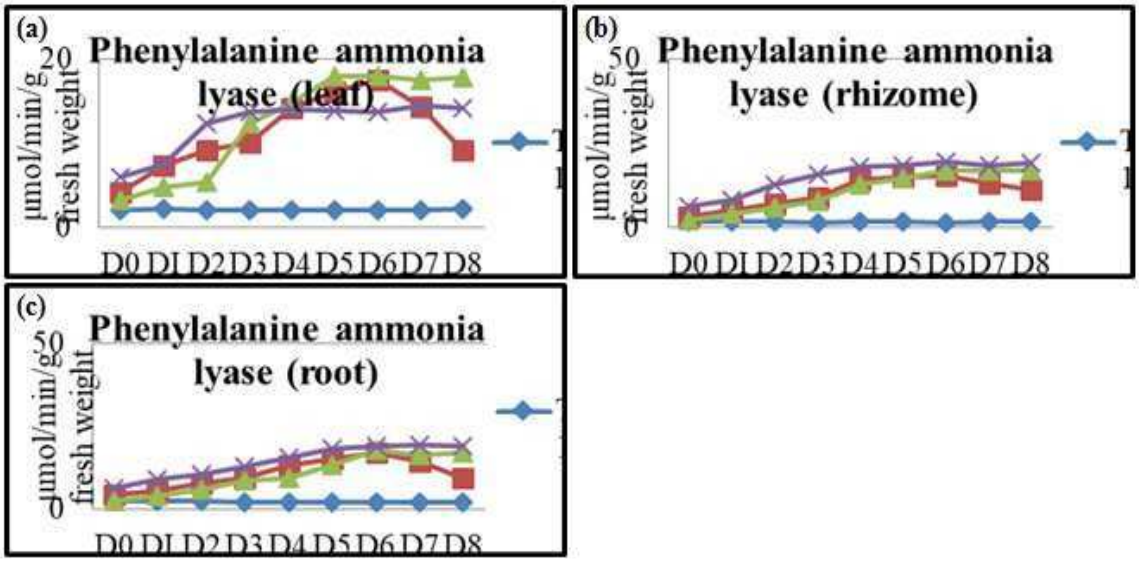

Figure 3: Change inPAL Activity in; (A)- Leaf, (B)- Rhizome and (C)- Root Tissues of Turmeric Treatments (LSD at 5% Level of Significance).

Assay of Lipoxygenase (LOX)

There is no significant difference in LOX activity was observed in any of the tissues of T1. In leaf tissues, lipoxygenase activity was significantly high in T3 lines, being recorded the highest activity on day 8 (10.44 folds) followed by T4 lines with highest activity noted on day 4 (8.74 folds). In rhizome tissues also there is no significant change in lipoxygenase activity except for a sparse change enhancement in day 6 (1.53 folds). Unlike leaf tissues, the T4 lines showed highest values of activities instead of T3 lines. In root tissues also there is no significant change in lipoxygenase activity of T2 lines while significantly high activities was observed with T4 lines from day 0 followed by T3 lines. In T4 lines, the highest activity was noted with day 6 (20.25 folds) and in T3 lines the enhanced activity was noted with 15.67 folds increase in day 7.

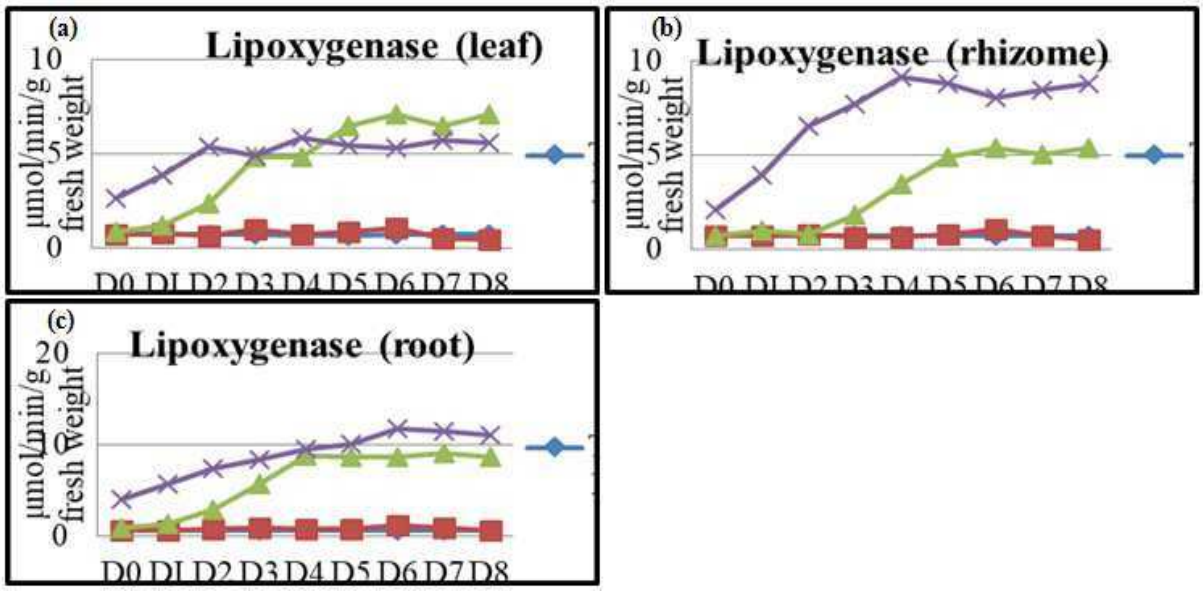

Figure 4: Change in LOX Activity in; (A)- Leaf, (B)- Rhizome And (C)- Root Tissues of Turmeric Treatments (LSD At 5% Level of Significance).

Assay of B (1, 3) Glucanase

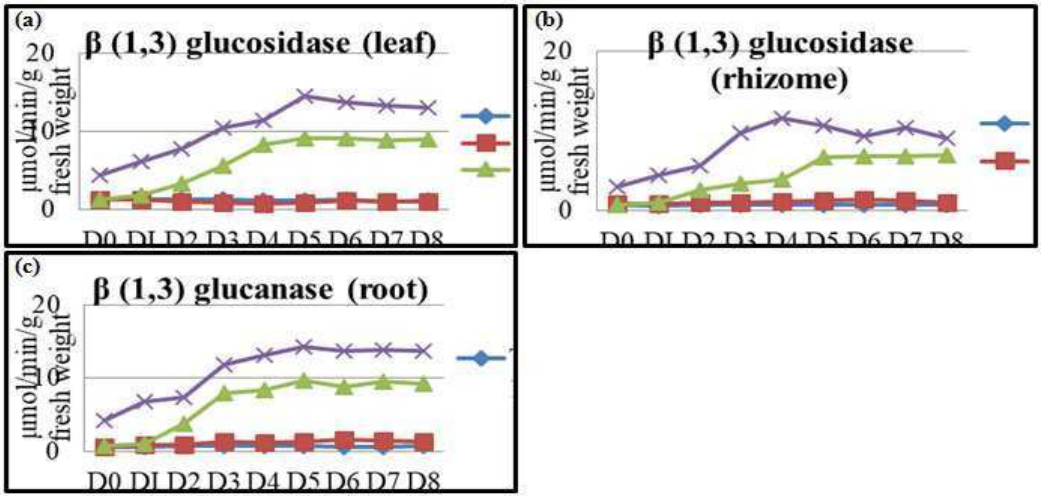

Figure 5: Change in Beta (1,3) Glucanase activity in;(A)- Leaf(B)- Rhizome and (C)- Root Tissues of Turmeric (LSD At 5% Level of Significance).

No significant change in β (1,3) glucanase activity was observed with T1 lines of all the three tissues. In leaf tissues, from day 0 onwards β (1,3) glucanase activity shot up compared to other treatment lines with the highest activity on day 5 with 12.14 fold increase as compared to absolute control. In T3 lines also, significant increase in enzyme activity was noted from day 1 onwards with highest activity on day 5 (7.66 folds). In rhizome tissues, highest activity was noted on day 4 (16.15 folds). Even though there are some abrupt changes in the β (1,3) glucanase activity of T4 lines the values remained significantly high throughout the experiment. T3 lines also showed a significant increase in enzyme activity from time to time with a maximum of 9.92 folds on day 7 as compared to the absolute control plants. Unlike leaf tissues, T2 lines of rhizome and root tissues showed a slight increase in β (1,3) glucanase activity as the experiment progresses. Highest β (1,3) glucanase activity was observed with T4 lines of root tissues with the highest value being 21.45 fold on day 6. In T3 lines also a significant increase in enzyme activity was noted on day 7 (14.61 folds).

Assay of Total Phenols

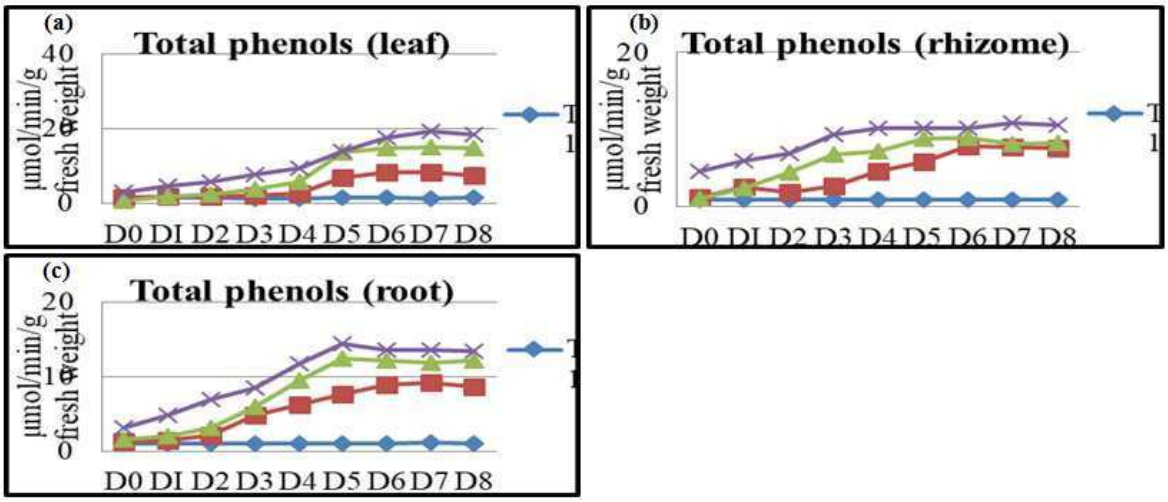

Figure 6: Change in Total Phenols I-in; (A)- Leaf, (B)- Rhizome and (C)- Root Tissues of Turmeric (LSD at 5% Level of Significance).

In leaf tissues, highest production was observed with T4 followed by T3 and then in T2. In T4 and T3 the highest content was observed on 7th day with 13.3 folds and 10.37 folds respectively. Upon pathogen inoculation, the total phenols all the tissues of T4 remained on the higher side from day 0 to day 8. In rhizome tissues and root tissues also phenol content was high in T4 lines followed by T3 and then in T2. In rhizome tissues, the highest increase was observed on day 8 in T4 (11.22 folds), day 5 (9.36 folds) in T3 and day 6 (8.05 folds) as compared to the T1. In root tissues, on 5th day T4 (14.43 folds) and T3 (12.46 folds) showed highest phenol contents.

3. DICUSSION AND CONCLUSIONS

The nitrogen fixing, phosphate solubilizing and potassium utilizing *B. cepacia* strain IISRCLRB5 showed 71.81 % inhibition *in vitro* and 100 % rhizome rot control in turmeric plants against *P. aphanidermatum in vivo* (Bijitha and Suseela, 2019). Early and elevated levels of expressions of various defence enzymes in response to pathogen attack and biocontrol agents are important features in inducing systemic resistance. The present study was focused to determine the changes in the levels of defence related enzymes and total phenols in turmeric plants in response to pathogen attack (*P. aphanidermatum*), biocontrol treatment alone (*B. cepacia* strain IISRCLRB5) and both together.

Peroxidase mediates lignifications by producing H_2O_2, its role in lignification is well studied in plants (Lamb & Dixon, 1997). As a primary line of defence against invading pathogens, lignin is involved in cell wall building processes. The oxido-reductive enzyme peroxidase is found to participate in phenol oxidation, lignification and suberization of host plant cells as a part of defense reaction against pathogens (Ray *et al,* 1998). POD induction is reported to be one of the early events in many plant-microbe interactions (Harrison *et al,* 1995). Upon inoculation with *Pseudomonas syringae,* peroxidase activity was increased in the first 24 hpi in cucumber plants (Smith *et al,* 1991). In this study also an increase in peroxidase activity was observed with pathogen challenging and rhizobacterial treatment. The highest activity was observed with T4 indicates the protective role played by the bacteria against *P. aphanidermatum* attack. The PPO activity is strongly correlated to high pathogen resistance (Raj *et al,* 2006). PPO induction following resistant plant-pathogen interactions have been reported in various pathosystems including wheat-*Fusarium graminearum* (Mohammadi and Kazemi, 2002), potato-*R.solanacearum* (Poiatti *et al,* 2009), *Pectobacterium atrosepticum*- potato (Ngadze *et al,* 2012), *Uromyces hobsoni-Jasminum officinale, R. solanacearum*-patchouli interactions (Xie *et al,* 2017). PPOs are also induced in susceptible interactions, such as potato soft rot and in non-host interactions, such as bacterial spot of citrus (Poiatti *et al,* 2009). In the present study also, high induction of PPO was observed on pathogen inoculation and an extensively high induction of the enzyme was observed with the involvement of rhizobacterium.

In various host-pathogen interactions, increase in PAL activities have been found *viz.*, *Eruca sativa*, *Alternaria brassicicola* (Gupta *et al,* 2014), tomato-*Fusarium oxysporum* (Mandal *et al,* 2009), tomato-*R. solanacearum* (Vanitha *et al,* 2009) interactions. Those transgenic tobacco plants were found to be more sensitive to fungal infection than wild-type plants when their PAL activities were suppressed (Maher *et al,* 1994) and they were unable to establish SAR in response to pathogen infection (Pallas *et al,*, 1996). PAL plays an important role in the synthesis of salicylic acid (Mauch-Mani and Slusarenko, 1996), a compound necessary for the induction of systemic acquired resistance in many plants (Dixon and Paiva, 1995). In our study also on rhizobacterium treatment, the PAL activity was found to be increased extensively, suggests the role of *B. cepacia* strain IISRCLRB5 in disease resistance through the induction of PAL. LOX are involved in metabolic route leading to the synthesis of various antimicrobial compounds such as JA and aldehydes involved in plant defence (Dixon and Paiva, 1995). Increase in LOX activity in response to pathogen infection has been reported for a

number of plant-pathogen systems (Vanitha and Umesha, 2008). Here the present study confirms the enhancement of LOX in response to pathogen invasion along with the protective role of *B. cepacia* strain IISRCLRB5 in disease resistance.

β -1,3-glucanase is involved in hydrolytic cleavage of the 1,3- β -D-glucosidic linkages in β -1,3-glucans of the of fungi and oomycetes cell wall (Hoj & Fincher, 1995; Borad & Sri ram, 2008). As a result of the hydrolysis of glucans in the cell walls of the pathogen, cell lysis and cell death occurs. The fungal elicitors released from pathogen cell wall can induce β -1, 3-glucanases in the different parts of the host plant (Borad & Sriram, 2008). These enzymes are found in a wide variety of plants like *Arachis hypogea*, *Cicer arietinum*, *Nicotiana tabacum* etc. and having resistance against various fungi like *Aspergillus parasiticus, A. flavus, Blumeria graminis, C. lagenarium, F. culmorum, F. oxysporum, F. udum, Macrophomina phaseolina* and *Streptomyces sioyaensis* (Borad & Sriram, 2008). This study confirms the role of β -1, 3-glucanases along with other defence related enzymes in pathogen invasion. Phenolics are important due to the formation of soluble and insoluble complexes by binding to proteins *in vitro* (Torti *et al*, 1995). These interactions play a major role in defensive function of phenolics and are key factors in the plant defense against fungal infection (Carrasco *et al*, 1978). Some plants may express these phenolic compounds as a means of passive defense constitutively **(**Barry & Manley, 1986), or they may be expressed on attack by pests and diseases (Grey *et al*, 1997). In this study also it was found that a small quantity of total phenol content even in absolute control lines which may suggests their constitutive expression. The content was high in T2 on pathogen challenging indicate the expression in response to pathogen attack. A high content was observed with T3 lines and the highest phenol content was observed with T4 indicates the protective role rendered by *Burkholderia cepacia* strain IISRCLRB5.

In all the tissues we studied, the defence related enzymes and total phenols are found to be significantly higher on pathogen challenging in *B. cepacia* strain IISRCLRB5 treated turmeric plants as compared with other treatment lines, indicates the crucial role of particular rhizobacteriain induction of important defence related enzymes. ISR helps plants to withstand pathogen attack specifically in roots or leaves, may not impart total protection (Haas and Défago, 2005). While our study clearly indicates that the rhizobacteria treatment enhances protection in rhizome tissues also. This finding along with the previous findings on the organism (Bijitha and Bhai 2019) increases the scope of usage of *B. cepacia* strain IISRCLRB5 as a biocontrol agent against rhizome rot of turmeric. But this could be established only after evaluation under field conditions.

4. ACKNOWLEDGMENTS

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